Pilot Scale Milling Characteristics of Transgenic Isolines of a Hard Wheat Over-Expressing Puroindolines

J. M. Martin,* F. D. Meyer, C. F. Morris, and M. J. Giroux

ABSTRACT

Wheat (*Triticum aestivum* L.) grain texture is an important determinant of milling properties and end product use. Two linked genes, puroindoline a (*Pina*) and puroindoline b (*Pinb*), control most of the genetic variation in wheat grain texture. Our goal was to examine milling characteristics of transgenic isolines of the hard red spring wheat cultivar Hi-Line overexpressing *Pina* (HGA), *Pinb* (HGB), or both (HGAB), which have soft (HGAB and HGB), intermediate (HGA), and hard (Hi-Line) grain texture. A second goal was to evaluate the flour quality of the genotypes for cookies and bread. Genotypes were grown in replicated trials in two environments. Grain was milled in a Miag Multomat pilot scale flour mill which closely emulates a commercial long flow mill. Stream yield and ash and protein content were determined from 10 flour and four bran streams. Cookie and bread quality was determined from straight grade flour. Break flour yield ranged from 404 g kg^{-1} for HGAB to 202 g kg^{-1} for Hi-Line. Straight grade flour yield ranged from 711 g kg^{-1} for HGAB to 744 g kg^{-1} for Hi-Line. Cumulative ash curves showed harder textured wheats (Hi-Line and HGA) had greater ash content from break streams, but more horizontal slope than soft wheats (HGAB and HGB) for the portion of the curve describing the relationship between ash and flour extracted from the endosperm. Flours from the soft isolines, HGAB and HGB, suffered less starch damage than flour from intermediate HGA or hard Hi-Line. Flours from HGAB and HGB were best suited for cookies. All three transgenic isolines overexpressing either or both puroindolines had smaller loaves of bread than Hi-Line. Puroindolines directly impact milling properties and may indirectly affect end use properties such as cookie properties and loaf volume by modifying water hydration traits.

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Abbreviations: HGA3, 'Hi-Line' overexpressing *Pina*; HGB12, 'Hi-Line' overexpressing *Pinb*; HGAB18, 'Hi-Line' overexpressing *Pina* and *Pinb*; *Pina*, puroindoline a; *Pinb*, puroindoline b; SKCS, single kernel characterization system.

Wheat is classified into hard and soft classes based on the texture of the grain. The distinction between soft and hard classes of wheat is governed mainly by the Hardness (*Ha*) locus on chromosome 5DS (Mattern et al., 1973; Law et al., 1978). Greenwell and Schofield (1986) identified friabilin as a marker protein for grain softness which was present in large amounts on the surface of water-washed starch of soft wheats and nearly absent from hard wheats (Bettge et al., 1995; Greenblatt et al., 1995; Morris et al., 1994). Friabilin is composed of two major polypeptides termed puroindoline a (PINA) and puroindoline b (PINB). Genes coding for these two proteins, *Pina* and *Pinb*, are tightly linked to the *Ha* locus on chromosome 5D (Sourdille et al., 1996; Giroux and Morris, 1997) and probably function together as the *Ha* locus (Giroux and Morris, 1998). Recent results have shown that mutations in either *Pina* or *Pinb* are associated with the expression of hard texture (Giroux and Morris, 1997, 1998; Lillemo and Morris, 2000; Morris et al., 2001). The glycine to serine mutation in *Pinb* (*Pinb-D1b* allele) and the null mutation for *Pina* (*Pina-D1b*

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allele) are the two most common mutations among U.S. hard wheats (Morris et al., 2001).

In wheat, transgenic expression of wild-type *Pinb-D1a* sequence in the hard spring wheat Hi-Line complemented the glycine to serine mutation (*Pinb-D1b* allele) resulting in a soft phenotype (Beecher et al., 2002). Hogg et al. (2004) expressed *Pina-D1a*, *Pinb-D1a*, or both in the same Hi-Line background. Expression of either *Pinb*, or both *Pina* and *Pinb* gave a soft phenotype, while *Pina* alone was intermediate in grain texture. Transgenic expression of wild-type *Pina-D1a* sequence in the hard wheat 'Bobwhite' which has the *Pina-D1b* (null) allele also gave a soft phenotype (Martin et al., 2006). The evidence from the transgenic experiments indicates that both wild-type *Pin* genes must be present to give the soft phenotype, and the soft phenotype can be restored by complementing either mutated *Pinb* or null *Pina* allele with the corresponding wild-type *Pin* allele. Swan et al. (2006) crossed Hi-Line transgenic lines expressing *Pina* or *Pinb* to a soft wheat and found progeny with added *Pinb* had softer grain than those with added *Pina*. They concluded *Pinb* may be more limiting than *Pina* to grain softness in soft wheats.

Flour milling performance is a key trait involved in the processing and use of wheat. The soft and hard texture classes coincide with dramatic differences in milling and end-use properties (reviewed in Pomeranz and Williams, 1990; Morris and Rose, 1996). Soft wheats require less energy to mill than hard wheats and yield a higher proportion of break flour, the flour released from the initial stages of milling. The milling process produces many free intact starch granules from soft wheats, whereas milling fractures many starch granules from hard wheats giving a higher proportion of damaged starch. As a result, soft wheat flours absorb less water than hard wheat flours. Because soft and hard wheat flours have different properties, they are usually targeted for different end-use properties. Soft wheats are best suited for cookies, cakes, and pastries, while hard wheats are used for bread and bread products.

Measures of milling quality and efficiency include flour extraction rate and ash content (Morris and Rose, 1996; Posner and Hibbs, 1997). Flour extraction rate has obvious economic implications. Ash content of flour is important to end-users as it is a crude proxy for bran contamination. Further, the ash content of individual mill streams provides an indication of milling performance and endosperm–bran separation. Ash content generally increases from the central endosperm to the outer bran layers. For this reason, ash content increases with flour extraction rate. The milling quality of a commercial lot of wheat is often judged by sorting ash content of mill streams in ascending order, then determining cumulative ash content and cumulative extraction rate for successive mill streams, and finally plotting the cumulative ash content versus cumulative extraction rate (Lillard and Hertsgaard, 1983). Such a curve can be used to predict ash content at various extraction rates as well as assess overall milling efficiency (Morris and Rose, 1996). Short flow experimental mills used for routine genotype evaluation preclude such a milling efficiency evaluation because samples from multiple mill streams are not collected.

Although physical grain characteristics such as kernel weight and size influence milling properties, grain texture is the overriding factor affecting milling characteristics. The direct effect grain texture has on milling quality is difficult to assess without controlling other genetic factors between hard and soft wheats. Hogg et al. (2005) evaluated the milling and bread baking characteristics using a short flow experimental mill for a set of transgenic isolines consisting of four lines with added *Pina*, eight lines with added *Pinb*, and five lines with both *Pina* and *Pinb* along with untransformed control hard wheat Hi-Line, which represented very soft to hard grain texture. They found softer grain texture was associated with lower total flour yield, but higher break flour yield. Flour ash was highly related to total flour yield ($r = 0.72$). Loaf volume was less for softer textured entries. The soft transgenic lines may have been disadvantaged because they are not well suited for bread baking, and milling characteristics may have been biased because all genotypes were tempered to a constant moisture level optimal for soft wheats and flour protein contents differed. However, the loaf volume reduction was still observed in Hogg et al. (2005) when whole wheat flour was used and protein content was similar.

These studies raise questions about the role of grain texture on milling efficiency and quality, and whether the milling characteristics observed with experimental short-flow milling procedures could be observed in pilot scale milling procedures. Our goal was to thoroughly examine milling characteristics of transgenic isolines of wheat overexpressing *Pina*, *Pinb*, or both and a control giving a range in grain texture from very soft to hard. We accomplished this by milling replicated samples of the genotypes in a pilot scale flour mill. A second goal was to evaluate the flour quality of the genotypes for cookies and bread.

MATERIALS AND METHODS

Hi-Line hard red spring (Lanning et al., 1992) wheat and three transgenic isolines (HGA3, HGB12, and HGAB18) were chosen for pilot scale milling evaluation. HGA3 overexpresses *Pina-D1a*, HGB12 overexpresses *Pinb-D1a*, and HGAB18 overexpresses both *Pina-D1a* and *Pinb-D1a*. The derivation and characterization of these transgenic isolines is described in Hogg et al. (2004, 2005). The four genotypes were grown in two replications of a randomized block design under both rainfed and irrigated conditions at the Arthur H. Post Field Research farm near Bozeman, MT. A plot was 12 rows, 25.6 m long for the rainfed trial and 15.2 m long for the irrigated trial, with rows 30 cm apart. Each plot was harvested with a plot combine. Approximately 36 kg of grain was obtained from each plot.

Grain texture, kernel weight, and kernel diameter were determined using the Perten Single Kernel Characterization System (SKCS) 4100 (Perten Instruments, Springfield, IL) on 100 seeds per replication. Grain protein content was determined by near-infrared spectroscopy for whole grain using the Tecator Infratec 1225 Grain Analyzer (Foss North America, Silver Spring, MD).

Pilot Scale Milling

Wheat was cleaned using a Clipper cleaner, which has an air lifting, followed by a no. 16 round screen and a 0.21 by 1.27 cm slotted screen, the middle fraction being accepted for milling. Approximately 31 kg of cleaned grain from each replicate was tempered by adding water. The first temper (up to 130 g kg^{-1} water for HGB12 and HGAB18 and 150 g kg^{-1} for HGA3 and Hi-Line) was added 24 h before milling. The wheat was given a second temper by the addition of 5 g kg^{-1} water 10 min before milling based on dry weight.

Each replicate was milled on a Miag Multomat pilot scale mill (Posner and Hibbs, 1997). The mill produces 10 flour streams and four feed streams from three break and five reduction rolls (Fig. 1). Feed rate was 920 to 980 g min^{-1}. Break rolls were adjusted so that material flow through the mill was balanced while still achieving good bran clean-up characteristics without excessive shattering. The target first break release was for 430 g kg^{-1} of the grind to pass through a no. 24 Tyler (707 μm) wire screen in 20 s of sifting. The target second break release was for 640 g kg^{-1} of the grind to pass through the above sifting. The third break roll was adjusted to clean the bran as completely as possible without excessive shattering. The adjustment for reduction rolls was done by observation of stock with the objective of making as much flour as possible by the end of the process, but not to the point of overgrinding and flaking the stock.

Each of the 10 flour streams and four feed streams was weighed. A sample from each stream was analyzed for moisture, ash, and protein content. Moisture content was determined using 2 to 3 g of each stream by heating in an aluminum dish in a convection oven for 1 h at 130°C (AACC Method 44–15A). Ash content was measured on 3- to 5-g samples ignited and heated for 18 h at 580°C in a muffle furnace (AACC Method 08–01). Protein content was determined using 0.25 g flour samples and a LECO FP-528 N analyzer (LECO Corp., St. Joseph, MI). Protein content was obtained as N in g kg^{-1} × 5.70 with flour protein corrected to a 140 g kg^{-1} moisture basis (AACC Method 46–30). Straight grade flour was a composite of the 10 flour streams blended together using a horizontal ribbon blender. Starch damage on straight grade flour was determined using AACC method 76–31 (AACC, 2000).

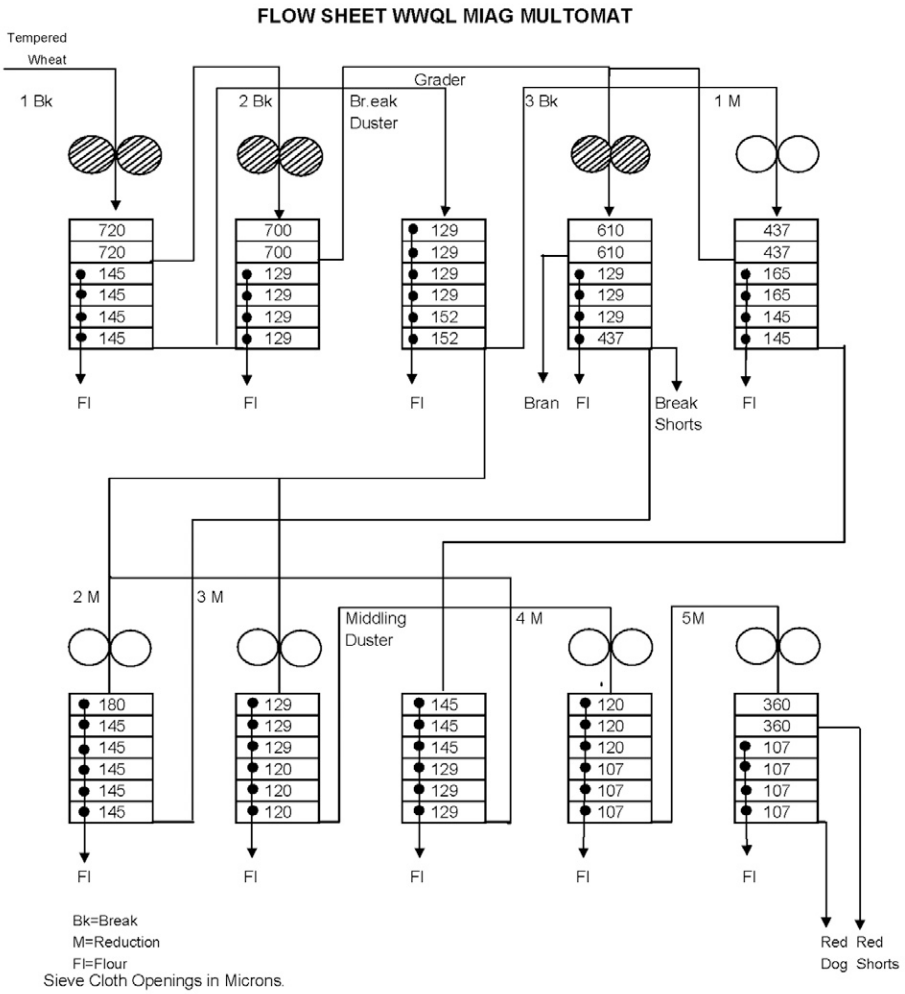

Figure 1. Flow diagram for the Miag Multomat pilot scale flour mill.

End Product Evaluation

End product evaluation was performed on flours from the four genotypes and on three flour mixtures formed by mixing three quarters Hi-Line straight grade flour with one quarter straight grade flour of each of the three transgenic isolines. Mixograph dough properties were evaluated using AACC method 54–40. Mixing time was time in minutes required for optimum dough development. Water absorption was determined visually by the swings of the mixograph curve and reported as concentration by weigh corrected to 140 g kg^{-1} flour. Mixograph type was visually evaluated on a 1 to 8 scale with higher values indicative of greater dough tolerance. Standard bake tests were conducted using AACC method 10–10B (AACC, 2000). Loaf volume was determined by the volume of canola seeds displaced. Crumb grain was scored on a visual 0 to 5 scale (5 = best) by an experienced baker. Sugar snap cookies were prepared using AACC method 10–52 starting with 40 g flour. Width and thickness measurements were obtained on two cookies per replication.

Data Analysis

Each response variable was analyzed via analysis of variance. Environment (rainfed and irrigated), genotype, and the interaction were treated as fixed effects using PROC GLM in SAS (SAS Institute, Inc., 2004). Means of the four genotypes were compared using the ESTIMATE statement in SAS. In addition,

Table 1. Means for grain yield and kernel characteristics for Hi-Line hard red spring wheat and transgenic isolines overexpressing puroindoline a (HGA3), puroindoline b (HGB12), or both puroindoline a and b (HGB18) averaged over two replications for rainfed and irrigated environments at Bozeman, MT.

Genotype	Grain yield	Grain hardness†	Kernel weight	Wheat protein	Kernel diameter
	kg ha^{-1}		mg	g kg^{-1}	mm
Hi-Line	5788	73.7	35.0	144	2.51
HGA3	5642	42.0	33.6	143	2.36
HGB12	5328	9.7	35.4	144	2.38
HGAB18	5645	6.4	34.3	147	2.35
P value‡	0.014	0.000	0.007	0.009	0.010
CV%	2.3	5.8	1.4	0.7	1.9
LSD(0.05)	232	3.3	0.8	2	0.08

†Obtained from Single Kernel Characterization System.

‡Genotype main effect *P* value.

for cookie and bread quality measurements, each flour mixture was compared to the weighted mean of the single components using the ESTIMATE statement in SAS.

Cumulative ash was computed by first ranking the 14 mill streams from lowest to highest ash content. The ash content of successive cumulative streams was computed on a total products basis. Cumulative flour extraction was computed from the ranked stream yields on a cumulative products basis (Lillard and Hertsgaard, 1983). The cumulative ash and cumulative extraction was computed for each replication for each genotype in each of the two environments. Ash and protein contents of break flour were computed in the manner described above, except only the three break streams were used. Ash and protein contents of straight grade flour were determined directly.

The relationship between cumulative ash and cumulative flour extraction was modeled using a linear segmented nonlinear model as outlined in Schabenberger and Pierce (2002). The model was:

$$\gamma_{ij} = (\beta_{0j} + \beta_{1j}x_{ij})I(x_{ij} \le \alpha_j) + [\beta_{0j} + \beta_{1j}\alpha_j + \beta_{2j}(x_{ij} - \alpha_j)]I(x_{ij} > \alpha_j)$$

where γ_{ij} is cumulative ash for genotype j, x_{ij} is cumulative flour extraction for genotype j, β_{0j} is an intercept for genotype j, β_{1j} and β_{2j} are slope coefficients for the two linear segments and α_j is the join point joining the two linear segments for genotype j. I is an indicator function that takes on a value of 1 if $x_{ij} > \alpha_j$ and 0 otherwise. The model parameters were estimated using PROC NLMIXED in SAS (SAS Institute, Inc., 2004). Differences between genotypes for individual parameters were compared using ESTIMATE statements.

RESULTS AND DISCUSSION

Genotype by environment interactions were not detected for grain yield or kernel traits, but additional irrigation did produce higher grain yield (6964 vs. 4237 kg ha^{-1}), lower grain protein content (137 vs. 152 g kg^{-1}), and greater seed weight (38.1 vs. 31.0 mg) for irrigated versus rainfed environments, respectively. HGB12 had significantly less grain yield than Hi-Line ($P < 0.05$), while the other two transgenic isolines did not differ from Hi-Line (Table 1). HGA3 was intermediate in grain texture between Hi-Line and HGB12 and HGA3B18. All genotypes were different from each other in grain texture except for the difference between HGB12 and HGAB18 ($P = 0.052$). These results agree with earlier reports for these same genotypes where addition of *Pinb* or both *Pina* and *Pinb* produced softest grain and addition of *Pina* produced intermediate grain texture (Hogg et al., 2004, 2005). For seed weight and grain protein, HGA3 had lower seed weight, and HGAB18 had higher grain protein content than Hi-Line. Otherwise transgenic lines were similar to Hi-Line. Hi-Line had larger diameter seeds than the three transgenic lines, while the three transgenic lines could not be differentiated for seed diameter.

Pilot Scale Milling

Differences among genotypes were detected for stream yield ($P < 0.01$) for all mill streams (Table 2). First break and second reduction had the highest mean yields among the flour mill streams (175 and 171 g kg^{-1}, respectively). These two streams showed greatest absolute difference among the four genotypes. For first break the extraction rates ranked opposite to grain texture with HGAB18 having highest and Hi-Line the lowest extraction. On the other hand, extraction rates for second reduction stream ranked the same as grain texture. The four genotypes also differed for the four streams comprising the bran fractions ($P < 0.01$). The bran stream accounted for the largest proportion (221 g kg^{-1}) on average of total product. Magnitude of differences among genotypes was greatest for this stream, with Hi-Line being least and HGAB18 greatest. Genotype interactions with environment were not detected for stream yields except for the second reduction stream.

Break flour yield ranged from 201 g kg^{-1} for Hi-Line to 404 g kg^{-1} for HGAB18 (Table 2). It is interesting that HGAB18 was clearly separated from HGB12 on the basis of break flour yield (360 vs. 404 g kg^{-1}), whereas the two genotypes were not for grain texture ($P = 0.052$). For straight grade flour yield, the softest genotypes, HGB12 and HGAB18, had lower flour yield than Hi-Line ($P < 0.01$) while HGA3 was intermediate, but less than Hi-Line

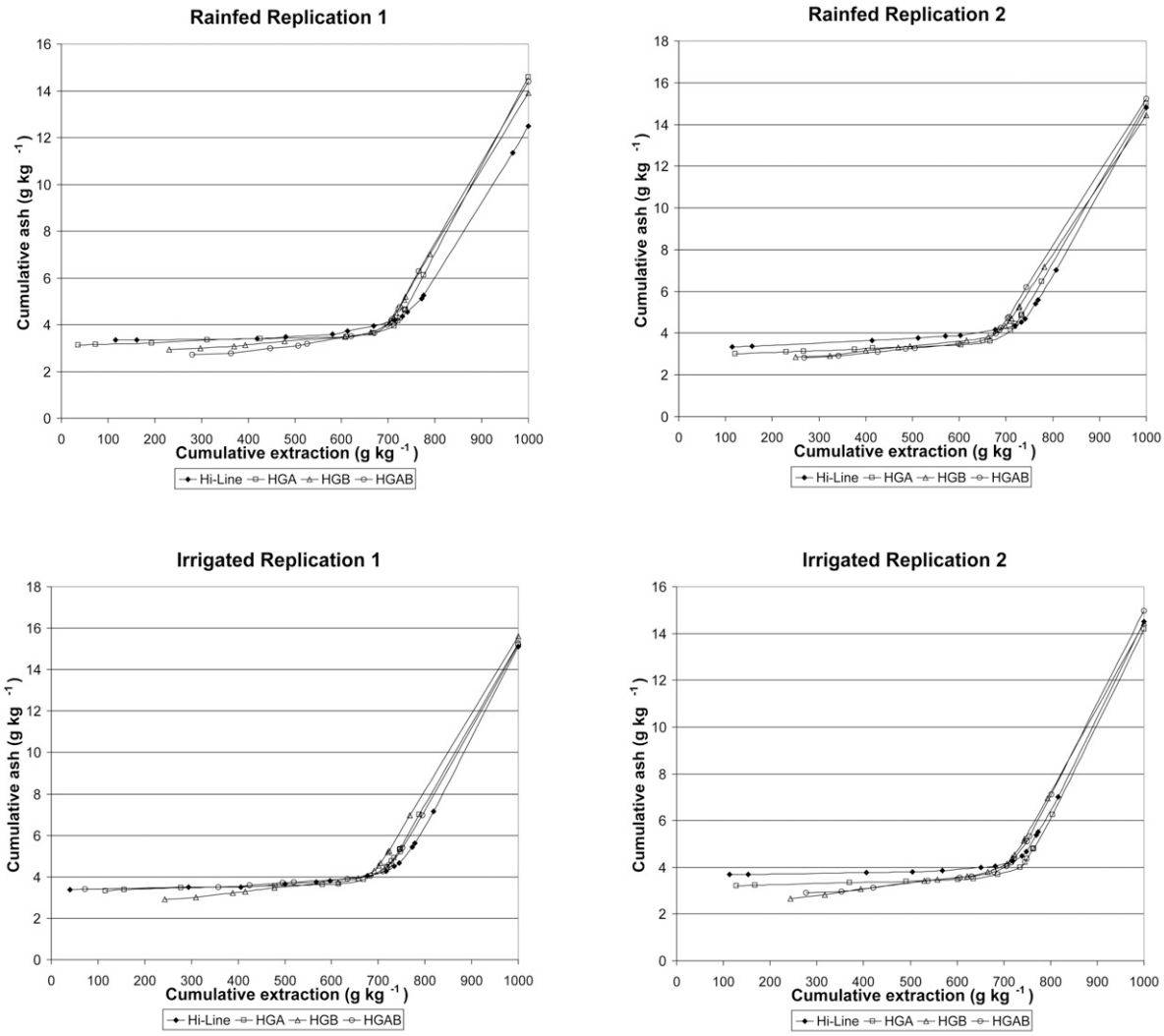

Figure 2. Plot of cumulative ash content versus cumulative flour extraction rate for Hi-Line hard red spring wheat and three transgenic isolines over-expressing puroindoline a (HGA), puroindoline b (HGB), or both puroindoline a and b (HGAB), from grain produced in two replications in rainfed and irrigated environments at Bozeman, MT.

($P = 0.049$). HGB12 and HGAB18 were similar in straight grade flour yield, whereas they were clearly separated for break flour yield. Genotypes performed similarly between environments for both break and straight grade flour yield, but the irrigated environment gave lower break flour yield (307 vs. 317 g kg^{-1}) but higher straight grade flour yield (731 vs. 719 g kg^{-1}) than the rainfed environment.

Hogg et al. (2005) found that when the same four genotypes were tempered to moisture content optimum for soft wheat and milled on a Quadrumat experimental mill, relative rankings were similar for flour yield. However, flour yields were lower, and the range between Hi-Line and HGB12 and HGAB18 was about 100 g kg^{-1}.

Protein content for mill streams tended to increase from the earlier (break fractions) to the later stages of milling (reduction and bran fractions) (Table 3). Within the break streams, protein content increased from first to third break, and genotype differences ($P < 0.05$) were observed for all three break streams. The four genotypes ranked in the same order for protein content and grain texture for first break where Hi-Line had highest and HGAB18 lowest protein content, but genotype rankings did not follow grain texture for the remaining two break streams. HGAB18 had higher protein than HGB12 for all three break streams. This may be related to higher grain protein content for HGAB18. Differences among genotypes were also detected for the grader, and third and fifth reduction streams. Hi-Line had lower protein than the three transgenic lines for third and fifth reduction streams, but the opposite was true for the grader stream. Protein content in both break and straight grade flour differed among genotypes, with HGB12 being lower than the other three genotypes. Genotype by environment interactions for protein content in mill streams were not observed.

Ash content was highest for mill streams making up the bran fraction (Table 4). Genotypes differed ($P < 0.01$) for all streams making up straight grade flour except first middling redust stream. In contrast genotype differences ($P < 0.01$) were observed for only one (red dog shorts) of the four streams from the bran fraction. Hi-Line was higher

Table 2. Stream yields from 14 streams from Miag pilot scale flour mill for Hi-Line hard red spring wheat and transgenic isolines overexpressing puroindoline a (HGA3), puroindoline b (HGB12), or both puroindoline a and b (HGB18) averaged over two replications for rainfed and irrigated environments at Bozeman, MT.

	Break streams				Reduction streams								Bran streams			
Genotype	B1†	B2	B3	Break flour	GR	M1	M1RD	M2	M3	M4	M5	Straight grade flour	BKSH	BRAN	RED-DOG	REDSH
								g kg^{-1}								
Hi-Line	63	98	41	202	31	114	45	255	71	17	10	744	38	187	26	4
HGA3	117	108	56	281	36	122	39	195	47	10	04	732	40	214	13	1
HGB12	242	72	47	360	70	75	26	130	38	10	05	714	49	217	17	3
HGAB18	278	77	49	404	76	64	22	105	28	08	04	711	42	224	20	3
P value‡	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.002	0.003	0.01	0.002
CV%	4.3	3.5	6.6	1.9	7.6	1.8	12.1	1.5	17.2	8.7	18.3	1.0	5.1	3.8	16.4	23.1
LSD(0.05)	13	5	6	10	7	3	7	5	14	2	2	11	4	14	5	1

†Streams are first, second, and third break (B1–B3); Grader (GR); First, second, third, fourth, and fifth reduction (M1–M5); first middling reducer (M1RD); break shorts (BKSH); bran (BRAN) red dog (REDDOG); and red dog shorts (REDSH).

‡Genotype main effect *P* value.

Table 3. Protein content of products from 14 streams from Miag pilot scale flour mill for Hi-Line hard red spring wheat and transgenic isolines over-expressing puroindoline a (HGA3), puroindoline b (HGB12), or both puroindoline a and b (HGB18) averaged over two replications for rainfed and irrigated environments at Bozeman, MT.

	Break streams				Reduction streams								Bran Streams			
Genotype	B1†	B2	B3	Break flour	GR	M1	M1RD	M2	M3	M4	M5	Straight grade flour	BKSH	BRAN	REDDOG	REDSH
								g kg^{-1}								
Hi-line	145	163	202	144	143	136	126	123	127	146	159	138	176	162	162	159
HGA3	129	164	198	145	137	108	114	124	160	180	197	137	182	163	151	141
HGB12	106	153	173	137	110	119	136	149	161	169	191	132	180	175	163	140
HGAB18	119	172	195	148	120	120	125	135	161	179	197	137	185	179	166	146
P value‡	0.000	0.032	0.003	0.022	0.000	0.22	0.122	0.071	0.000	0.066	0.049	0.004	0.108	0.082	0.177	0.059
CV%	4.2	4	3.4	2.4	2.7	13.7	8.6	9.2	3.6	9.3	8.9	2.4	2.4	5.1	5.1	5.6
LSD(0.05)	9	11	11	6	6	28	19	21	10	27	29	11	8	15	14	14

†Streams are first, second and third break (B1–B3); Grader (GR); First, second, third, fourth, and fifth reduction (M1–M5); first middling reducer (M1RD); break shorts (BKSH); bran (BRAN) red dog (REDDOG); and red dog shorts (REDSH).

‡Genotype main effect *P* value.

in ash ($P < 0.01$) than the three transgenic lines for first and third break, break flour, and grader streams, yet that trend was reversed for third, fourth, and fifth reduction streams. Although differences among the transgenic lines were observed, that pattern of differences did not always follow a consistent trend across mill streams. HGB12 and HGAB18 did not differ from each other ($P < 0.05$) for any mill stream except for third break and fourth and fifth reduction streams. Despite differences in ash content for nearly all individual mill streams, genotypes did not differ in ash for straight grade flour. This apparent inconsistency is because relative rankings of genotypes changed across mill streams. Genotypes performed relatively the same for ash content between environments for individual mill streams.

Morris et al. (1946) and later Hinton (1959) showed ash content followed a gradient increasing from central to outer layers of the endosperm. Highest ash content was in the bran fractions of wheat. Break flour results from the initial breaking of the kernel and release of flour from the central portion of the endosperm. Later stages of milling give flour from outer portions of the endosperm which are more likely to contain bran contaminants depending on efficiency of the milling process. Soft genotypes HGB12 and HGAB18 gave their lowest ash content for first break, but harder genotypes HGA3 and Hi-Line gave their lowest ash content at first reduction stream.

The relationship between ash content and flour extraction was characterized by plotting cumulative ash content versus cumulative flour extraction for the four genotypes in each replication from the two environments (Fig. 2). The shapes of the curves for a given genotype were consistent within replications and environments. The lone exception was one replication from the irrigated environment for HGAB18. The lower segment represented ash content primarily from endosperm, while the upper segment represented ash content from bran fractions. The relationship was formalized with a segmented linear regression model. The linear slopes for the lower segment ranged from 0.1047×10^{-2} for HGA3 to

Table 4. Ash content of products from 14 streams from Miag pilot scale flour mill for Hi-Line hard red spring wheat and transgenic isolines overexpressing puroindoline a (HGA3), puroindoline b (HGB12), or both puroindoline a and b (HGB18) averaged over two replications for rainfed and irrigated environments at Bozeman, MT.

Genotype	Break streams			Break flour	Reduction streams							Straight grade flour	Bran streams			
	B1†	B2	B3		GR	M1	M1RD	M2	M3	M4	M5		BKSH	BRAN	REDDOG	REDSH
									g kg^{-1}							
Hi-Line	4.26	4.15	8.12	4.27	5.21	3.49	4.19	3.65	5.1	12.94	17.71	4.93	38.13	45.54	25.63	34.91
HGA3	3.40	3.67	5.82	3.71	3.81	3.25	3.36	3.66	10.16	22.54	29.30	4.85	34.77	45.30	27.88	31.62
HGB12	2.84	4.36	6.39	3.81	3.26	3.95	4.43	4.52	11.57	20.89	27.37	4.93	33.76	41.91	27.56	31.39
HGAB18	2.99	4.18	5.87	3.78	3.21	4.07	4.66	4.76	11.01	18.63	22.90	4.65	34.48	43.94	26.05	33.58
P Value‡	0.000	0.004	0.000	0.000	0.001	0.011	0.202	0.006	0.002	0.000	0.000	0.176	0.2	0.317	0.63	0.001
CV%	7.6	3.8	4.3	1.9	10.2	6.8	18.9	8.1	14.2	3.1	8.2	3.1	7.6	6.2	10.6	2.2
LSD(0.05)	0.44	0.27	0.48	0.13	0.69	0.44	1.36	0.58	2.32	0.99	3.45	0.23	4.62	4.77	4.90	1.23

†Streams are first, second, and third break (B1–B3); Grader (GR); First, second, third, fourth, and fifth reduction (M1–M5); first middling reducer (M1RD); break shorts (BKSH); bran (BRAN) red dog (REDDOG); and red dog shorts (REDSH).

‡Genotype main effect *P* value.

0.2535×10^{-2} for HGB12 (Table 5). We found that the two softest genotypes, HGB12 and HGAB1818, had greater slope than Hi-Line ($P < 0.05$), while HGA3 did not differ from Hi-Line for the lower segment. Linear slopes for the upper segment ranged from 3.489×10^{-2} for HGB12 to 3.809×10^{-2} for HGA3. None of the three transgenic lines had slopes different from Hi-Line for the upper segment. The join points between the two segments ranged from 695 g kg^{-1} for HGB12 to 729 g kg^{-1} for Hi-Line. The breakpoint occurred at a lower extraction rate for all three transgenic lines compared to Hi-Line ($P < 0.01$). The join points are in the same rank order, but slightly less than straight grade flour yields. We found the four genotypes did not differ for wheat ash meaning the cumulative ash curves had essentially the same ash content at 100% extraction (i.e., whole grain meal). A desirable curve, indicating excellent milling quality, would be one with low ash and minimal slope for the lower segment, with the join point occurring at a high extraction rate. Clearly, the soft genotypes, HGB12 and HGAB18, have lowest ash for low flour extraction rates. This reflects the higher proportion of low ash break flour fractions for these genotypes. The response for HGA3 is interesting in that it is more horizontal than that for the two soft genotypes, and intersects at about 555 g kg^{-1} extraction, giving lower ash beyond that point. The response for Hi-Line always lies above the three softer transgenic lines.

Ash content in straight grade flour did not differ among the four genotypes, yet HGB12 and HGAB18 had greatest linear slope for the lower portion of the ash curves than did Hi-Line. This points out the soft genotypes had lower ash content from initial stages of milling, and that relative differences in ash content varied widely from different stages of milling. First break and second reduction are examples. We do not believe Hi-Line has higher ash content in the central endosperm. Rather the higher ash content for Hi-Line compared to HGB12 and HGAB18 for first break is probably because flour arose from center to outer layers of endosperm in Hi-Line rather than primarily from the central endosperm as in HGB12 and HGAB18. This reflects the more efficient milling for the softer HGB12 and HGAB18 genotypes. A concurrent difference in flour protein was also observed for first break stream indicating this stream for Hi-Line was comprised of layers throughout the endosperm.

Cumulative ash curves are used in the commercial milling industry to assess milling efficiency and quality (Morris and Rose, 1996) and to predict milling characteristics of new cultivars before release (Morris and Engle, unpublished data, 2006). Most often they are presented without characterizing the curve. Lillard and Hertsgaard (1983) used a cubic regression and a linear regression to describe lower and upper portions. Flores et al. (1991) used a segmented cubic–linear nonlinear model to estimate

Table 5. Estimates of coefficients from segmented linear/linear nonlinear model for relation between cumulative ash and cumulative flour extraction for Hi-Line hard red spring wheat and transgenic isolines overexpressing puroindoline a (HGA3), puroindoline b (HGB12), or both puroindoline a and b (HGB18).

Genotype	Intercept		Lower segment		Upper segment		Join point	
	b_0	SE	$b_1 \times 10^{-2}$	SE $\times 10^{-2}$	$b_2 \times 10^{-2}$	SE $\times 10^{-2}$	a	SE
Hi-Line	3.24	0.15	0.1096	0.0280	3.630	0.072	729	4
HGA3	3.01	0.13	0.1047	0.0280	3.809	0.070	714	4
HGB12	2.17	0.21	0.2535	0.0425	3.489	0.065	695	4
HGAB18	2.40	0.20	0.2396	0.0385	3.676	0.067	701	4

the cubic and linear regression parameters as well as the breakpoint. We used a linear–linear segmented nonlinear model because of its simplicity and ease of comparing parameters. Although cumulative ash curves have been presented (Li and Posner, 1989; Posner and Deyoe, 1986), we are not aware that formal genotype comparisons of curve properties have been reported.

End Product Evaluation

The proportion of damaged starch for the four genotypes followed expected trends with grain texture (Table 6). The hard wheat Hi-Line suffered the most starch damage, followed by intermediate-textured HGA3, and soft-textured HGB12 and HGAB18 having the least amount of damaged starch (Table 6). Starch granules are easily dislodged from the protein matrix of soft wheats. In contrast, starch granules are tightly bound and require more energy to remove from the protein matrix in hard wheats, and consequently are more prone to fracture during milling due to stronger granule-matrix bond.

The flours differed ($P < 0.01$) for both cookie weight and thickness but less so for diameter ($P = 0.061$) (Table 6). Flours from the softest genotypes, HGAB18 and HGB12, produced cookies with less mass and thickness than Hi-Line. The HGB12 and HGAB18 genotypes could not be distinguished for diameter or thickness. When transgenic and Hi-Lines flours were blended, they performed in an additive fashion for weight, diameter and thickness.

Cookie diameter is often used as a predictor of soft wheat quality. Gaines (2004) found cookie diameter was influenced by flour protein quantity and quality and grain texture. More specifically cookie diameter was negatively correlated with flour protein content and lactic acid solvent retention capacity, a measure of glutenin strength, but positively correlated with indicators of grain texture such as break flour yield. Since these genotypes are transgenic isolines, glutenin characteristics should be the same across genotypes. Although we did not assay glutenin patterns, Beecher et al. (2002) showed that storage proteins were not altered by the overexpression of *Pinb-D1a* in Hi-Line. Hi-Line was selected and released for its bread quality characteristics (Lanning et al., 1992). The desired glutenin characteristics for bread making may be detrimental for cookies. The protein quality characteristics fixed in these genotypes may be more limiting for cookie diameter than the grain texture differences among these genotypes.

Among the dough and bread quality traits, the seven flours differed for mixograph time ($P < 0.05$) and mixograph absorption and loaf volume ($P < 0.01$) (Table 6). Hi-Line flour absorbed more water than HGA3 ($P < 0.05$) and HGB12 and HGAB18 ($P < 0.01$). Similarly, Hi-Line produced larger loaves than HGA3 ($P < 0.05$) and HGB12 and HGAB18 ($P < 0.01$). The two softest transgenic lines, HGB12 and HGA3B18, did not differ in mixograph absorption and loaf volume. There was no evidence that blends of transgenic and Hi-line flours produced synergistic or antagonistic effects on dough or bread quality traits. The lone exception was for HGA3 added to Hi-Line for mixograph absorption. We did not detect differences ($P < 0.05$) for mixograph tolerance or crumb grain score. Genotypes performed similarly between the two environments as genotype by environment interactions were not important ($P < 0.01$). Hogg et al. (2005) found overexpressing *Pina*, *Pinb* or both reduced loaf volume averaged over several lines per transgene group compared to Hi-Line. Mixograph water absorption was less than the hard wheat control for the group overexpressing both *Pina* and *Pinb*, but not for the groups overexpressing *Pina* or *Pinb* alone. Hogg et al. (2005) included the same four genotypes as the current study. In their study the three transgenic lines each had loaf volume less than Hi-Line, but the four genotypes were not statistically different ($P < 0.05$) for mixograph water absorption. Their results were in part confounded by differences in flour yield which resulted in flour protein differences. Loaves baked from whole wheat flour where protein was equivalent still showed reductions in loaf volume for the HGA3 and HGAB18 lines but not the HGB12 line.

Dubreil et al. (1998) found that reconstituting flours lacking *Pina* with puroindolines enhanced loaf volume and improved crumb grain in a mixture of good and poor flours but not in the good or poor quality flours alone. They attributed the increase in loaf volume to a more optimal balance between tenacity (resistance to dough deformation) and extensibility. We saw no synergistic or antagonistic effect when flour from lines overexpressing puroindolines was mixed with Hi-Line. Martin et al. (2006) transgenicly expressed *Pina-D1a* sequence in a hard wheat with *Pina* null (Pina-D1b) allele. The soft transgenic isolines showed varying results for loaf volume. Two had loaf volume significantly less, but a third had loaf volume equal to the hard wheat control (Martin and Giroux, unpublished data, 2006).

Campbell et al. (2001) found that segregation at *Pinb* did not affect loaf volume in a soft by hard wheat recombinant inbred population, but water hydration traits such as starch damage and water absorption were affected. On the other hand, Martin et al. (2001) found *Pinb-D1b* gave greater loaf volume than *Pina-D1b* in a hard spring wheat population segregating for the two alleles even though the *Pinb-D1b* group was about 6 units softer than the *Pina-D1b* group.

Our study differs from Hogg et al. (2005) in that the four genotypes were tempered to follow commercial practices and thereby better optimize flour extraction based on grain texture. Also, we used a semicommercial scale mill. As a result flour protein among the genotypes was similar except for HGB12 which was lower than the other three. Flour protein differences do not offer an adequate explanation for the reduced loaf volume observed in the transgenic lines. It is not clear whether the changes in end use proper-

Table 6. Starch damage, cookie and bread quality traits made from straight grade flour for Hi-Line hard red spring wheat and transgenic isolines overexpressing puroindoline a (HGA3), puroindoline b (HGB12), or both puroindoline a and b (HGB18) and blends of Hi-line and each transgenic line averaged over two replications for rainfed and irrigated environments at Bozeman, MT.

Entry		Cookie			Mixograph			Bread	
	Starch damage	Weight	Diameter	Thickness	Tolerance[§]	Time	Absorption	Loaf volume	Crumb grain score[¶]
	%	g	cm	cm		min	g kg^{-1}	mL	
Hi-Line	6.70	40.0	7.8	1.73	5.8	4.4	647	1304	4.0
HGA3	5.01	39.3	8.0	1.68	5.3	4.1	638	1246	3.8
HGB12	2.35	39.5	8.1	1.50	5.5	4.1	629	1199	4.0
HGAB18	2.20	39.4	8.0	1.46	4.3	3.7	631	1193	4.0
HGA3+Hi-Line[†]		39.9	7.9	1.68	5.3	4.1	653	1286	3.8
HGB12+Hi-Line		39.9	7.9	1.61	5.5	4.1	639	1293	4.0
HGAB18+Hi-Line		39.8	8.0	1.59	5.3	4.3	646	1315	4.0
P Value[‡]	0.000	0.000	0.061	0.001	0.184	0.031	0.000	0.000	0.567
LSD(0.05)	0.64	0.1	0.2	0.11	1.1	0.4	8	46	0.4
CV%	14.4	0.13	1.24	4.26	13.6	6.1	0.8	2.3	6.8
Comparisons of blend vs. individual components									
HGA3+Hi-Line blend vs. components		0.708	0.595	0.406	0.427	0.245	0.022	0.871	0.29
HGB12+Hi-Line blend vs. components		0.205	0.742	0.221	0.688	0.205	0.257	0.442	1.00
HGAB18+Hi-Line blend vs. components		0.080	0.165	0.125	0.789	0.729	0.304	0.061	1.00

[†]Blend of 3/4 Hi-Line plus 1/4 transgenic line straight grade flours.

[‡]Entry main effect *P* value.

[§]Measured on 1 to 8 scale.

[¶]Measured on 0 to 5 scale with 5 being best.

ties such as cookie properties and loaf volume are due to puroindolines themselves or to the effects on water hydration traits brought about by changes in grain texture.

CONCLUSIONS

Transgenic overexpression of either or both puroindolines in a hard wheat gave grain texture phenotypes from very soft to hard. These transgenic isolines provided a unique means to determine the direct effect these genes have on milling characteristics and end product quality. This was accomplished through pilot scale milling of replicated samples of each genotype from two environments. The pilot scale mill closely emulates a long flow commercial mill. Mill stream yields conformed to expectations for genotypes with varying grain texture. The softest genotypes which overexpressed *Pinb* and both *Pina* and *Pinb* had a higher proportion of break flour, while harder genotypes, the untransformed control, and a genotype overexpressing *Pina*, showed greatest stream yields from later reduction stages. In addition the soft genotypes gave lowest ash content for streams from initial stages of milling, while harder genotypes had lowest ash content from later reductions stages. This points to the greater adherence of starch granules to the protein matrix for hard textured wheats, giving rise to more efficient extraction of flour from the central endosperm with less starch damage for soft wheats. When milling efficiency was compared from cumulative ash curves, the major differences occurred among genotypes when relating ash to flour extracted from endosperm, but not from bran fractions. End product evaluation showed soft genotypes were best suited for cookies while harder genotypes were better suited for bread.

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